RESEARCH COMMUNICATION

# Mutations of *KRAS* and *TP53* in a Minor Proportion of *Opisthorchis viverrini*-Associated Cholangiocarcinomas in a Hamster Model

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## Abstract

**Background/Aims *KRAS* oncogene and *TP53* tumor suppressor gene have been known as common genes involving in many cancers including cholangiocarcinoma (CCC). Activation of these genes could lead to uncontrolled proliferation and cancer ultimately. The aim of this study was to investigate mutation of *KRAS* exon 1 and *TP53* exon 5-8 in *Opisthorchis viverrini* (OV)-induced cholangiocarcinoma (CCA) in a hamster model. Methods: Twenty-seven CCAs were obtained from Syrian golden hamsters induced by OV infection and N-nitrosodimethylnitrosamine (N-NDDM) administration. The tumor tissues were processed for histopathology. Genomic DNA extracted from paraffin sections by microdissection was amplified for KRAS exon 1 and TP53 exon 5-8 mutations by PCR-direct sequencing. Results Histopathologically, the tumors were classified into tubular (81.5%, 22/27), papillary (3.7%, 1/27), mucinous (3.7%, 1/27) and mixed types (11.1%, 3/27). Of the 27 CCAs, PCR-direct sequencing of KRAS showed G‡A transition at codon 37 exon 1 in one CCA sample (3.70%). Point mutations of p53 exon 6 (G‡C transversion at codon 119 and 218 and A‡C transversion at codon 217) were found in 3 CCA samples (11.1%). Conclusions: The results suggest that mutation of TP53 particularly at exon 6 may be involved in cholangiocarcinogenesis and a novel mutation of *KRAS* exon 1 was firstly reported in OV-induced hamster CCA.**

**Key Words**: Cholangiocarcinoma - hamster - mutation - *KRAS* - *TP53* - *Opisthorchis viverrini*

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## Introduction

*Opisthorchis viverrini* (OV)-associated cholangi ocarcinoma (CCA) is major public health problem in Thailand, particularly in the Northeast (Sripa et al., 2007). OV infection is acquired by eating raw or uncooked cyprinoid fish harboring infective metacercariae. The fluke can induce host's pathology either by mechanical and immunopathological processes (Sripa, 2003) including inflammation, epithelial desquamation, epithelial and adenomatous hyperplasia, goblet cell metaplasia, and fibrosis of the hepatobiliary system. Inflammation of the bile ducts can induce oxidative DNA damage of the biliary epithelial cells leading to malignant transformation (Sripa et al., 2007). Moreover, endogenous or exogenous nitrosation could produce genetic and epigenetic alterations (Vatanasapt et al., 1999). CCA can be induced by OV infection and sub-carcinogenic dose of dimethylnitrosamine administration in hamsters (Thamavit et al., 1978; Thamavit et al., 1987). Molecular pathology of liver fluke associated CCA in human has been described (i.e., Kiba et al., 1993; Jinawath et al., 2006). However, genetic alteration of OV-induced CCA in the hamster model has not yet been reported.

*KRAS* is a member in the G-protein family. The mutated RAS gene appears to be oncogenic since its RAS protein could reduce GTPase activity and constitutively bind to GTP as described in various human cancers (Sherbet and Lakshmi, 1997). Mutation of this gene in human cancers was frequently found at codon 12, 13 and 61 with the most common at codon 12 (Bos, 1989). In human CCA, there are discrepancies in the mutation rates in different reports that may be from specimen collection and/or detection methods (Levi et al., 1991). Ohashi et al. (1996) reported *KRAS* mutation at codon 12 in 8 cases [GGT (Gly) to GAT (Asp) mutation and 2 cases of GGT to GTT (Val)] of 22 intrahepatic CCAs. By using sequence-specific oligonucleotide hybridization, Imai et al. (1994) reported 69% (16 of 23) of mutations were G→A transition (aspartic transition) at the first position of codon 12 (GGT). Other mutations were the transition to AGT (Ser), TGT (Cys), GAT, GCT (Ala) and GTT.

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**Table 1. *KRAS* and *P53* Primer Sequences by Chang et al. (1994) and Yamanaka et al. (1997) and PCR Conditions**

| Primers | Exon | Sequences | Conditions |
|---|---|---|---|
| HamkI | 1 | 5'-GGCCTGCTGAAAATGACTGA-3' | initial denaturation 95°C, |
| HamkY | | 5'-GTCCTGCACCAGTAATATGC-3' | 2 min; 30 cycles: 95°C 30 sec 55°C 1 min,72°C 1 min |
| Hap53-I4 | 5 | 5'-TCATCAGCTCCAACTCTGACCCTG-3' | initial denaturation at 94°C, |
| Hap53-I5A | | 5'-AAGAGCAATCAAGAACATCAACGG-3' | 5 min; 30 cycles: 95°C 30 |
| Hap53-I5 | 6 | 5'-CAATTAGAAATGCTTGCCTGGGGC-3' | sec, 60°C 30 sec, 72°C 1 |
| Hap53-I6A | | 5'-AGTCTGGGGTAGAGCAAACTAAAC-3' | min; 72°C 5min |
| Hap53-I6 | 7 | 5'-GTTACCATCAGTGTCCTTACTACA-3' | |
| Hap53-I7A | | 5'-AACACGCCAACAGGAACACAGCAA-3' | |
| Hap53-I7 | 8 | 5'-CTTACTGTCTCGTGCTCTCCCTCC-3' | |
| Hap53-I8A | | 5'-TGAAGCTGAACCTCCTCCTCTGCC-3' | |

Although *KRAS* mutation by using RFLP analysis has been reported in 17% in 24 OV-associated CCAs from Thailand (Wattanasirichaigoon et al., 1998), Tsuda et al. (1992) did not find any mutations of *KRAS* in Japanese and Thai patients. In addition, Petmitr et al. (1998), using PCR-SSCP and direct sequencing, could not detect any mutations in 20 CCAs from Thai patients. The low incidence of KRAS mutation from those studies may be duse to DNA samples from the whole tumor or the fixing duration (Levi et al., 1991; Cerny et al., 1992). In animal model, *KRAS* mutation has been shown to be a frequent and early event in experimentally non-liver fluke induced CCA in hamsters, particularly point mutations (G→A transition) at codon 12 (Cerny et al., 1992; Erill et al., 1996). Moreover, the mutation increases concomitantly with advance in malignancy.

TP53 is an important tumor suppressor gene in which genetic alterations have been commonly described in several cancers. In animal models, TP53 mutations have been frequently reported in hamster pancreatic cancer such as G→A transition occurs at codon 248 (Chang et al., 1996). TP53 mutation was observed in hamster pancreatic cell line but not in transplantable tumors (Cerny et al., 1992). No report on *KRAS* and TP53 mutations in OV-induced CCAs has been described so far in animal model. We therefore aimed to study *KRAS* exon 1 and TP53 exon 5-8 mutations in OV-associated CCA in hamsters.

## Materials and Methods

*Tumor induction*

Twenty seven, male, 3-4 weeks old Syrian golden hamsters (*Mesocricetus auratus*) were treated with *N*-nitrosodimethylnitrosamine (NDMA) 12.5 ppm, PO for 8 weeks and co-infected with 50 OV metacercariae by intragastric intubation (Thamavit et al., 1987). The animals were subsequently fed with normal diet and tap water *ad libitum* for 5-6 months post infection. The tumors were harvested 5-6 months post-treatment.

*Histopathological study*

The animals were sacrificed and their livers were fixed in 10% buffered formalin for 24 hours prior to routine tissue processing. Four-micrometer H&E stained sections were histopathologically evaluated. The tissue blocks having tumors were selected for microdissection.

*Microdissection and extraction of DNA*

Ten-micrometer-thick sections were obtained from the tumor paraffin blocks. Deparaffinization was made through immersing the sections in xylene for 10 min and then boiling in xylene for another 10 min. Next, the sections were hydrated, stained with Mayer's hematoxylin and dehydrated in graded concentration of ethanol. Under a light microscope, the sections were compared to their corresponding H&E stained samples to locate the representative tumor lesion. Sterile needles were used to precisely collect the tumor cells.

Genomic DNA from microdissected samples was extracted by the commercial DNA purification kit for paraffin-embedded tissue (PUREGENE®, USA). Briefly, each sample was placed into a 300 µl of the cell lysis solution. 1.5 µl of proteinase K solution was added into the solution before incubating at 55°C overnight. 1.5 µl of RNAase solution (4 mg/ml) was added into the sample and incubated at 37°C for 30 min. Protein precipitation solution was added to precipitate protein. After mixing well, the sample was centrifuged at 13,000xg for 3 min.

The supernatant was collected, mixed with 100% isopropanol and glycogen, and then spun down. The pellet was washed with 70% ethanol, air dried and DNA samples were finally rehydrated with DNA hydration solution.

*PCR and direct sequencing of KRAS Exon 1 and TP53 Exons 5-8*

Primer sequences for *KRAS* mutation described by Yamanaka et al (1997) were adopted for the detection of mutation at the first two bases of *KRAS* exon 1. Primer sequences specific to *TP53* exon 5-8 described by Chang et al. (1994) were used. The primer sequences and conditions for DNA amplification with Perkin Elmer 2400 thermocycler are shown in Table 1. The PCR products from the 2% agarose gel electrophoresis was eluted by QIAquick, gel extraction kit (QIAGEN, Germany) or cleaned up by Hiyield Gel/PCR Minikit (Real-Biotec, Taiwan). The eluted products were sequenced by an automatic sequencing (ABI prism 3100,, Applied Biosystem, USA).

## Results

*Histopathology of hamster CCA*

Histopathological examination revealed that the

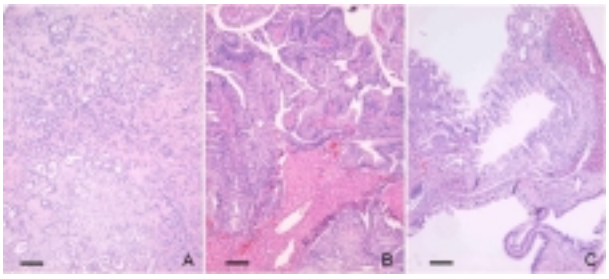

**Figure 1. Histopathological Types of Hamster CCAs.** Tubular (A), papillary (B), mucinous (C) (bar = 50 mm)

majority of hamster CCAs were tubular type (81.5%, 22/27) and minority were papillary (3.7%, 1/27), mucinous (3.7%, 1/27) (Figure 1) and mixed adenocarcinomas (2 tubular type with cystadenocarcinoma and 1 with mucinous type) (11.11%, 3/27). Proliferative (bile duct hyperplasia) and precancerous lesions (bile duct dysplasia and cholangiofibrosis) were also observed.

*Mutation of KRAS exon 1 in hamster CCA*

The *KRAS* product size was approximately 169 bps. Both forward and reverse sequences were analyzed and compared to GenBank No. AF285779 [17]. From 27 hamster CCAs, mutation was found only in 1 case (3.70%) at codon 37 of exon 1 (Figure 2). The first base changed from G (GAG) to A (AAG) (G→A transition) results in the change of amino acids from glutamic acid (Glu/E) to lysine (Lys/K). This mutation was similar to that in OV-induced hamster CCA cell line (HalCCA-1) (Sripa et al., unpublished).

*Mutation of TP53 in hamster CCA*

The PCR products of *TP53* exons 5-8 were 335, 236, 226 and 220 bps, respectively. The sequencing data of these products revealed distinct mutation in exon 6 in 3 of 27 CCA samples (Figure 3). None of the hamster CCAs showed mutation of *TP53* exons 5, 7 and 8.

For exon 6, 3 of 27 CCAs showed different sites of point mutation (Figure 3). The first case (2DMN1) showed double peak of bases G and C at codon 218 (c.653G>C). This peaks reflects G→C transversion which results in amino acid changing from AGT (ser/S)→ACT (threonine/T) or S218T. Nucleotide 596 (codon 199, c.596G>C) in the second case (No.2DMN25) showed G→C transversion which results in the change of amino acid from CGA (arginine/A) to CCA (proline/P) or R199P. The last case (2DMN27) showed abnormal A→C transversion of nucleotide 650 (codon 217, c.650A>C) leading to the change of amino acid from CAC (histidine/H) to CCC (proline/P) or H217P. These three hamster CCAs showed different mutations without specific pattern of *TP53* exon 6 mutations and did not relate to any histological types.

## Discussion

Liver fluke induced CCA in hamster is a useful model in cancer research, particularly in the study of the progression of the tumor. In this study we reported a relatively few mutation rates of both *KRAS* and *TP53* genes in OV-induced hamster CCAs.

Hamster *KRAS* gene possesses a very high degree of homology with the corresponding *KRAS* in other rodent species and human (Hesketh, 1994). Our study reported *KRAS* mutation at codon 37 of exon 1. There has been no report of G→A mutation (c.109G>A) of *KRAS* leading to amino acid changes from E→K (E37K) either in human or hamster CCA. This finding is different from other reports of mutation at codons 12, 13 and 61 which affects nucleotide binding capacity and hydrolysis (Hesketh,1994). The study of RAS structure by Hall et al. (2002) indicated that the amino acid E37 in switch I (residue 25-40) participated in stabilizing the switch and imposing sterical constraints. This interaction prevents nucleotide release through guanine exchange factors (GEFs) which promote GDP dissociation and subsequent GTP entrance. Mapelli et al. (2005) hypothesized that disruption of a hydrogen bond network involving the residues including E37 in switch I region could change

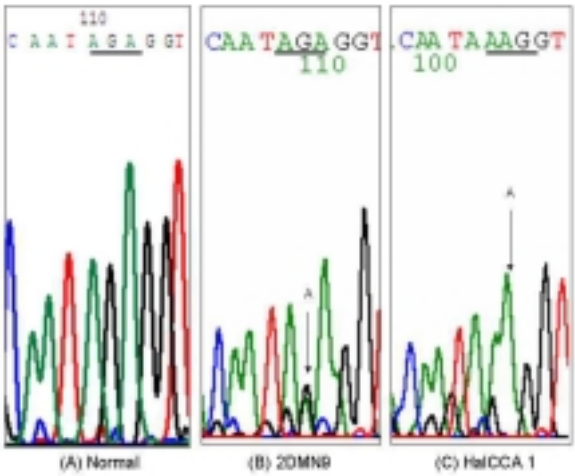


**Figure 2. Chromatograms of Normal (A), CCA (B, 2DMN9) and Mutated (C, HalCCA1) KRAS.** Heterozygosity of both G and C peak at the same position was showed in B (arrow). E37K transition was found in hamster CCA cell line (C). Amino acid residues were changed from glutamic acid (AGA) to lysine (AAG)

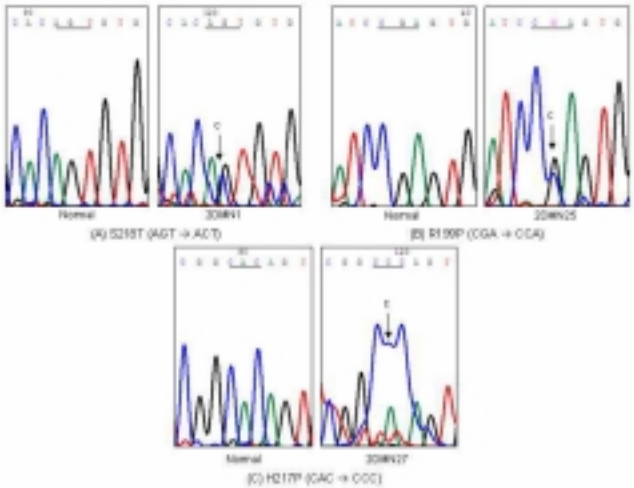

**Figure 3. Chromatograms Depicting Normal Sequence and Point mutation of TP53 Exon 6 in CCAs. A**mino acid changes at codon 218 (S218T) (A, 2DMN 1) and 199 (R119P) (B, 2DMN25) was due to G→C transversion but that at codon 217 (H217P) (C, 2DMN27) was due to an A→C transversion

**Table 2. Mutations in Hamster CCAs Comparing to Human Cancers (source: http://www.umd.be:2072/IFAMTP53.shtml, http://www-p53.iarc.fr/TumorCriteria.ASP and Khan et al., 2005)**

| Case number | Mutation in hamster CCA | Mutation in human | Reports in human tumors | Location | Reports in human CCA |
|---|---|---|---|---|---|
| 2DMN1 | S218T (c.653G>C) | S215T (c.644G>C) | 4 records in bladder, esophagus, glioblastoma, skin | S7 | Thailand (Kiba et al., 1993) |
| 2DMN25 | R199P (c.596G>C) | R196P (c.587G>C) | 17 records in bladder, breast, ung,Li-Fraumeni syndrome | S5 | None |
| 2DMN27 | H217P (c.650A>C) | H214P (c.641A>C) | 1 record and found in construction for functional analysis | S7 | Korea (Kang et al., 1999) |

conformation of the protein leading to switch I displacement and consequent guanine nucleotide release. Substitution of codon 37 of exon 1 serving as "effector domain" region reduces the biological effect of RAS protein but not GTP binding or hydrolysis. Interaction of mutated RAS proteins with cellular targets involves the stimulation of GTPases activity by GTPase activating protein (GAP) (Hesketh, 1994). By this manner, mutation of *RAS* exon 1 at codon 37 may play active role in proliferation, induce dramatic increases in active form of RAS protein, activate and lead cell into DNA synthesis, subsequently promote cell proliferation and tumor progression (Ohashi et al., 1996).

Studies of tumors other than CCA in Syrian golden hamster presented high frequency of *KRAS* mutation (Cerny et al., 1990; van Kranen et al., 1991; Cerny et al., 1992; Yamanaka et al., 1997; Fujii et al., 2005). Cerny et al. (1990) reported *KRAS* mutation in transplantable pancreatic ductal adenocarcinoma in hamster. They found G→A transition in the second position of codon 12 and 13. Mangold et al (1994) were able to transform hamster pancreatic duct cells in vitro by uses of *N*-(2-hydroxypropyl) nitrosourea or MNU and BOP–induced tumors. All samples were identified to have KRAS mutation at codon 12 or 13. However, the incidence was low in short schedules treatment with MNU. This indicates that other genes might get involved in carcinogenesis. With the used of BOP-induced CCA, Yamanaka et al (1997) detected *KRAS* mutation in a part of intrahepatic duct hyperplasia of which suggested that the *KRAS* mutation was an early event. The induction of extrahepatic biliary hyperplasia in hamsters by cholecystoduodenostomy and BOP administration showed a G→A transition in the second position of codon 12 (Majima et al., 1997).

Our result on *KRAS* mutation is different from those reported in human and animals which showed mutations mainly on codon 12, 13 and 61. Tada et al. (1992) reported 56% (9/18) of CCAs contained the mutations while human CCA from OV endemic area showed less *KRAS* mutation (Tsuda et al., 1992; Kiba et al., 1993). The chemical-based study of *KRAS* mutation by Yamanaka et al (1997) revealed higher percentage of KRAS mutation at codon 12 in CCA of papillary type than of tubular type. The mutation usually involved G:A→C:T transition. *KRAS* mutation found in our study may be mainly affected by NDMA because G→A substitution is the typical pattern of the major promutagenic DNA adductions of alkylating compounds ($O^6$-alkyldeoxyguanosines) occurring during replication. Transitions are also generated by 8-hydroxy-deoxyguanosine which is produced by oxygen free radicals, exogenous genotoxic agents, endogenous or nitric oxide-mediated deammination of cytosine and methyl cytosine (Loeppky, 1994). Accordingly, we would advocate that this type of *KRAS* mutation was first found in hamster CCA.

Hamster and human *TP53* sequences have a high homology (Legros et al., 1992). Moreover, *TP53* mutations in hamster are highly similar to those in human when compared to human *TP53* database (http://www.umd.be:2072/IFAMTP53.shtml, http://www-p53.iarc.fr/TumorCriteria.ASP). The database suggests that the mutation may occur in DNA binding region which probably altered its binding function (Table 2). It is clear that the DNA-binding domain is more susceptible to inactivation by amino acid substitution than the NH2- and COOH-terminal domain especially the two α-helices or 11 β-strands (Kato et al., 2003). It has been postulated that *TP53* formed a tetramer with a *TP53* binding site to activate the expression of adjacent genes and subsequently inhibited growth and/or invasion (Vogelstein and Kinzler, 1992). This missense mutation would result in reduction of functionally active tetramer through DNA binding. The mutated sites in our study (R199P, H217P, S218T) which correspond to those in human (R196P, H214P, S215T) locate on b-sheet (S5 for R196P, S7 for H214P, S215T) of *TP53*. Mutations of these areas may result in global unfolding leading to structural change and reduction of functionally active tetramer through DNA binding (Friedler et al., 2002). Mutation in the regions is responsible for the DNA binding domain of the protein which affects binding capacity and leads to accumulation of protein.

Mutation of many genes including *KRAS* and *TP53* has some linkage with chemical carcinogens (Harris, 1996). Our study has shown, especially, *TP53* mutations in exon 6 that may implicate the mutagenic effects of both OV and NDMA. It has been known that NDMA is a strong alkylating and methylating agent. It generates $N^7$-methylguanine ($N^7$-meG) and $O^6$-methylguanine ($O^6$-meG) (Kyrtopoulos, 1998). $N^7$-meG is not a direct pre-mutagenic but has capability to form mutagen. In contrast, $O^6$-meG is a strong and direct mutagenic which play a major role in carcinogenesis. NDMA fed hamsters

developed high levels of N-meG and even higher levels of $O^6$-meG in both liver and CCA (73% incidence) (Bosan et al., 1987). Even $O^6$-meG results in G:C→A:T transitions during replication, there are some case reports of human cancers that etiological involvement of NDMA are not dominated by G:C→A:T transitions. This probably relates to environment, prolonged duration and low-dose of NDMA exposure (Kyrtopoulos, 1998).

In conclusion, cellular and molecular pathogenesis of OV-induced CCA may be from chronic inflammation caused by OV infection together with mutagenic effects of NDMA. Mutation of *TP53* particularly exon 6 involves in cholangiocarcinogenesis in our study. Moreover, this study reports the first mutation of *KRAS* exon 1 at codon 37 in hamster CCA.

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